

Please type a plus sign (+) inside this box ☐

03-09-00

PTO
U.S.
09/521527
03/08/00

UTILITY
PATENT APPLICATION
TRANSMITTAL

Attorney Docket No.

ORT1199

First Named Inventor or Application Identifier

Didier Leturcq

Express Mail Label No.

EL457890296US

(only for new nonprovisional applications under 37 CFR 1.53(b))

APPLICATION ELEMENTS

ADDRESS TO:

Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

See MPEP Chapter 600 concerning utility patent application contents.

1. ☒ Fee Transmittal Form (attached hereto in duplicate)
2. ☒ Specification [Total Pages 18]
(Preferred arrangement set forth below)
 - Descriptive Title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R&D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☐ Drawing(s) (35 USC 113) [Total Sheets]
4. Oath or Declaration
 - a. ☐ Newly executed (original or copy)
 - b. ☒ Unexecuted original
 - c. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional check boxes 5 and 16)
 - i. ☐ Deletion of Inventor(s)
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation by Reference
(useable if Box 4c is checked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4c, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
 - a. ☐ Computer Readable Copy
 - b. ☐ Paper Copy (identical to computer copy)
 - c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement
(when there is an assignee) ☐ Power of Attorney
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement
(IDS)/PTO-1449 ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
14. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)

15. ☐ Other:

16. ☐ If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

Amend the specification by inserting before the first line: -- This is a ☐ Continuation ☐ Divisional
☐ Continuation-in-Part (CIP) ☒ Non-Provisional of prior application No.: 60/124,253, filed March 12,

1999. --

17. For this divisional application, please cancel original Claims of the prior application before calculating the filing fee.

18. CORRESPONDENCE ADDRESS

☐ Customer Number or Bar Code Label

or ☒ Correspondence Address below

Name: Audley A. Ciamporcero, Jr., Esq.

Address: Johnson & Johnson
One Johnson & Johnson Plaza
New Brunswick, NJ 08933-7003 USA

19. TELEPHONE CONTACT

Please direct all telephone calls or telefaxes to Alan J. Morrison at:

Telephone: (732) 524-3592 Fax: (732) 524-2808

19. SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

NAME Alan J. Morrison

Reg. No. 37,399

SIGNATURE

DATE March 8, 2000

FEE TRANSMITTAL	<i>Complete if Known</i>	
	Application Number	Not Yet Known
	Filing Date	Herewith
	First Named Inventor	Didier Leturcq
	Group Art Unit	Not Yet Known
	Examiner Name	Not Yet Known
	Attorney Docket Number	ORT 1199

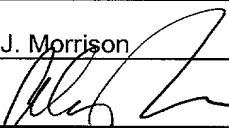
FEE CALCULATION

CLAIMS AS FILED

(1)	(2)	(3)	(4)	(5)
FOR:	NUMBER FILED	NUMBER EXTRA	RATE	BASIC FEE \$690.00
TOTAL CLAIMS	15- 20 =	0	x 18.00	\$ 0.00
INDEPENDENT CLAIMS	3 - 3 =	0	x 78.00	\$ 0.00
MULTIPLE DEPENDENT CLAIMS	<input type="checkbox"/>	N/A	\$260.00	
			TOTAL FEES	\$ 690.00

METHOD OF PAYMENT

- ☒ Please charge Deposit Account No. 10-0750/ORT1199/AJM in the amount of \$690.00. Three copies of this sheet are enclosed.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required in connection with the filing of this communication, or credit any overpayment, to Account No. 10-0750/ORT1199/AJM. Three copies of this sheet are enclosed.

SUBMITTED BY:		<i>Complete (if applicable)</i>
Typed or Printed Name	Alan J. Morrison	Reg. No. 37,399
Signature		Deposit Account No. 10-0750
	Date: 3/8/2000	

000000 22512300

IN THE UNITED STATES
PATENT AND TRADEMARK OFFICE

Applicant: Didier Leturcq

For : Method Of Isolating CD8+ Cells, And Related
Hybridoma Cells, Antibodies And Polypeptides

Express Mail Certificate

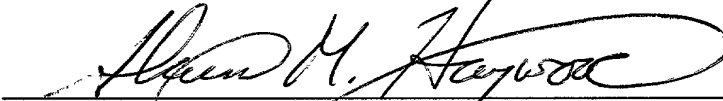
"Express Mail" mailing number: EL457890296US

Date of Deposit: March 8, 2000

I hereby certify that this complete application, including specification pages, claims, abstract and unsigned Declaration, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Alwin Haywood

(Typed or printed name of person mailing paper or fee)



(Signature of person mailing paper or fee)

000000 225123650

METHOD OF ISOLATING CD8⁺ CELLS, AND RELATED HYBRIDOMA CELLS,
ANTIBODIES AND POLYPEPTIDES

5 Throughout this application, various publications are
cited. The disclosure of these publications is hereby
incorporated by reference into this application to describe
more fully the state of the art to which this invention
10 pertains.

Field of the Invention

15 This invention relates to a positive selection method
for isolating CD8⁺ cells using certain CD8-specific
antibodies. The isolated CD8⁺ cells have importance as
vehicles for combating viral infections and tumors.

Background of the Invention

20 In humans, CD8⁺ cells play a vital role in the immune
system's ability to defend against potentially harmful
foreign entities, such as bacteria and viruses [1]. CD8⁺
cells circulate in the blood and possess on their surface
25 the CD8 protein. When necessary, these cells are
converted into cytotoxic cells (i.e. cell-killing cells)
which proceed to destroy foreign cells, viruses, and other
harmful pathogens present in the subject [2]. Because of
CD8⁺ cells' effective role in host defense, they hold
30 great potential in isolated form as therapeutics for
treating disorders such as viral infections and
malignancies [3].

35 In the past, purification of human CD8⁺ cells has
been achieved by negative selection. Specifically,

peripheral blood mononuclear cells ("PBMC's") are incubated with a cocktail of monoclonal antibodies specific for non-CD8 sub-populations. These sub-populations include, for example, B-cells, CD4⁺ cells, NK
5 cells, macrophages and neutrophils, and each contains specific, non-CD8 "markers". The sub-populations are then removed from the resulting antibody cocktail using magnetic beads [4]. This technique has certain major disadvantages. The first is that several monoclonal
10 antibodies are required for removing non-CD8⁺ cells. The second is that the resulting CD8⁺ population suffers from contamination from non-CD8⁺ cells that possess relatively low levels of non-CD8 markers. Finally, when a magnetic separation procedure is used to remove all non-CD8⁺ cells,
15 a large number of magnetic beads are needed.

Summary of the Invention

This invention provides a method of isolating CD8⁺ cells which comprises the steps of

- 5 (a) contacting a sample of isolated peripheral mononuclear blood cells with a first antibody which specifically binds to the sequence AAEGLDTQRFSG, or portion thereof, on CD8 molecules present on the surface of CD8⁺ cells but does not activate the CD8⁺ cells once bound thereto, under conditions permitting the formation of a first complex between the CD8⁺ cell and first antibody;
- 10 (b) separating from the sample any first antibody not present in the resulting first complex;
- 15 (c) contacting the sample with a second, immobilized antibody which specifically binds to the first antibody in the first complex, under conditions permitting the formation of an immobilized, second complex between the first complex and the second antibody, thereby immobilizing the CD8⁺ cells present in the sample;
- 20 (d) separating from the resulting immobilized second complex the cells present in the sample which were not immobilized in step (c);
- 25 (e) contacting the immobilized second complex under suitable conditions with an agent which causes the dissociation of the second complex into CD8⁺ cells and an immobilized third complex between the first antibody and second antibody; and
- 30 (f) separating the immobilized third complex from the CD8⁺ cells, thereby isolating the CD8⁺ cells.

[illegible]

10

15

- 20

- 25

- 30

- (d) separating from the resulting immobilized second complex the cells present in the sample which were not immobilized in step (c);
- 5 (e) contacting the immobilized second complex under suitable conditions with an agent which causes the dissociation of the second complex into CD8⁺ cells and an immobilized third complex between the first antibody and second antibody; and
- 10 (f) separating the immobilized third complex from the CD8⁺ cells, thereby isolating the CD8⁺ cells.

As used herein, a "CD8⁺ cell" means a T-cell having on its surface the CD8 protein. In the preferred embodiment, the CD8⁺ cells are human CD8⁺ cells. The CD8⁺ cells can be from any CD8⁺ cell-possessing species. "Isolating" CD8⁺ cells means obtaining a population of peripheral mononuclear blood cells wherein the ratio of CD8⁺ cells to non-CD8⁺ cells is at least about 7:1. In 20 the preferred embodiment of this invention, this ratio is at least about 9:1.

This invention employs several types of antibodies which specifically bind to given epitopes. More 25 particularly, this invention uses a "first antibody" which specifically binds to the sequence AAEGLDTQRFSG, or portion thereof, on CD8 molecules present on the surface of CD8⁺ cells but does not activate the CD8⁺ cells once bound thereto. Here, CD8⁺ cell "activation" means causing 30 CD8⁺ cells to express γ -interferon (" γ -IFN"). This activation can be measured using routine methods such as sandwich ELISA assays, which can be performed using commercially available kits.

Such first antibodies include, but are not limited to, the monoclonal antibodies produced by the hybridoma cell lines 37B1 (ATCC Accession No. HB-12441) and 8G6 (ATCC Accession No. HB-12657). Conditions which permit these antibodies to bind to but not activate CD8⁺ cells are well known in the art. These conditions are described, for example, in a suitable buffer such as Ca²⁺ and Mg²⁺-free Dulbecco's Phosphate Buffer Saline (DPBS) containing 1% Human Serum Albumin (HSA) and 0.2% sodium citrate and gentle mixing by "end over end" rotation on a rotator set at 4 rpm.

As used herein, the term "antibody" includes, but is not limited to, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and binding fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. In one embodiment, the antibody is a monoclonal antibody. The monoclonal antibody can be human, or that of another species including, for example, mouse and rabbit. In this invention, an antibody which "specifically" binds to a stated epitope binds to that epitope with a dissociation constant of at least about 10-fold less than the dissociation constant with which it binds to any other epitope. In one embodiment, this dissociation constant ratio is at least about 100. In the preferred embodiment, this dissociation constant ratio is at least about 10³.

The "second antibody" used in the instant method can be any antibody which specifically recognizes an epitope on any portion of the first antibody. In the preferred embodiment, the second antibody specifically recognizes a

0080E0" 225T25560

This invention further provides a population of CD8⁺ cells isolated by the instant method.

5 Finally, this invention provides a kit for use in isolating CD8⁺ cells which comprises, in separate compartments, (a) an antibody which specifically binds to the sequence AAEGLDTQRFSG, or portion thereof, on CD8 molecules present on the surface of CD8⁺ cells, but does
10 not activate the CD8⁺ cells once bound thereto; and (b) an agent which causes the dissociation of a CD8⁺ cell-antibody complex. In one embodiment, the agent which causes the dissociation of a CD8⁺ cell-antibody complex comprises the polypeptide having the sequence
15 AAEGLDTQRFSG. In the preferred embodiment, the agent is the polypeptide consisting of the sequence AAEGLDTQRFSG.

20 The instant kit can further comprise reagents useful for performing the binding and dissociation steps of the instant method. The components of the instant kit can either be obtained commercially or made according to well known methods in the art. In addition, the components of the instant kit can be in solution or lyophilized as appropriate. In the preferred embodiment, the kit further
25 comprises instructions for use.

30 The following procedures relating to the instant invention are routine in the art: isolating peripheral mononuclear blood cells from which the CD8⁺ cells are in turn isolated [6]; separating unbound antibodies and cells from a sample containing bound antibodies and/or cells via centrifugation or spinning membrane; and immobilizing antibodies via polystyrene flasks, columns or beads [4,7].

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Rationale

5 Human CD8⁺ cells can be isolated from preparations of
peripheral blood mononuclear cells (PBMC's) by either
positive or negative selection. Positive selection
results in a highly-purified population of CD8⁺ cells.
Negative selection, while resulting in sufficient numbers
10 of CD8⁺ cells, has low levels of contaminating non-CD8
populations remaining after the selection procedure.

The idea was to generate an antibody which has high
affinity for CD8⁺ cells, does not activate the cells
15 during the selection process, and is capable of being
easily eluted from the cells. An anti-peptide antibody
appeared to meet these criteria. However, it was known
that anti-peptide antibodies might be of low affinity and
may recognize the linear peptide sequence exclusively,
20 preventing reactivity with native antigen.

It was necessary that the anti-CD8 antibody not
activate the cells during the selection process, as it
would lessen their ability to effectively act as naïve
25 responder cells during *in vitro* stimulation protocols.
The use of peptide release to selectively isolate a cell
population has been shown by Tseng-Law, et al. [8] for
CD34⁺ cells.

Methods

The CD8 alpha chain was examined for hydrophilic sequences and four peptides selected. All were coupled to keyhole limpet hemocyanin (KLH) as carrier and used to immunize mice. A C-terminal amino acid was added to each of the peptides coupled to KLH to make the monoclonal antibodies. Antisera from the mice were evaluated for the ability to recognize both peptide and native CD8 on the surface of T cells. Only one of the four peptides was capable of recognition of both antigenic forms of CD8. Monoclonal antibodies were generated to this peptide and the resulting antibody used to isolate CD8⁺ cells from a PBMC preparation. The antibody was successful in isolating a population of highly-purified CD8⁺ cells (Table 1) which were not activated by the isolation procedure (Table 2).

Table 1

5 Purification of CD8⁺ Cells by
Positive Selection Analyzed by Flow Cytometry*

CELL TYPE	PEMC + Fluorescence (Range)	POST SELECTION + Fluorescence (Range)
CD8 T cells	15 (7-24)	82 (56-95)
CD4 T cells	36 (14-52)	2 (0.1-10)
CD14 Monocytes	15 (7-26)	0.8 (0.2-2)
CD15 Neutrophils	12 (8-21)	0.6 (0.1-3)
CD19 B cells	2 (0.4-7)	3 (0.5-9)
CD56 NK cells	6 (2-17)	6 (0.1-20)

* Summary of 10 normal donors

Table 2

10 Activation of CD8⁺ Cells Isolated By Negative or
Positive Selection (Assessed by IFN γ Production)

Cells	Negative Selection (pg/ml)	Positive Selection (pg/ml)
un-stimulated	20	20
allo-stimulation	1440	3600

References

1. Nabholz M. and H.R. MacDonald (1983) Annual Review of Immunology 1:273-306.
5
2. Riddell S.R. and P.D. Greenberg (1994) Current Topics in Microbiology and Immunology 189:9-34.
3. Riddell S.R. and P.D. Greenberg (1995) Annual Review of Immunology 13:545-586.
10
4. Horgan K and S. Shaw (1994) Current Protocols in Immunology 2:7.4.1.
- 15 5. Lea T, et al. (1988) Journal of Molecular Recognition 1(1):9-18.
6. Kanof, M.E., et al. (1994) Current Protocols in Immunology 2:7.1.1.
20
7. Kanof M.E. (1994) Current Protocols in Immunology 2:7:3:1.
8. PCT International Publication No. WO 95/34817.
25

What is claimed is:

1. A method of isolating CD8⁺ cells which comprises the steps of
 - 5 (a) contacting a sample of isolated peripheral mononuclear blood cells with a first antibody which specifically binds to the sequence AAEGLDTQRFSG, or portion thereof, on CD8 molecules present on the surface of CD8⁺ cells but does not activate the CD8⁺ cells once bound thereto, under conditions permitting the formation of a first complex between the CD8⁺ cell and first antibody;
 - 10 (b) separating from the sample any first antibody not present in the resulting first complex;
 - 15 (c) contacting the sample with a second, immobilized antibody which specifically binds to the first antibody in the first complex, under conditions permitting the formation of an immobilized, second complex between the first complex and the second antibody, thereby immobilizing the CD8⁺ cells present in the sample;
 - 20 (d) separating from the resulting immobilized second complex the cells present in the sample which were not immobilized in step (c);
 - 25 (e) contacting the immobilized second complex under suitable conditions with an agent which causes the dissociation of the second complex into CD8⁺ cells and an immobilized third complex between the first antibody and second antibody; and
 - 30 (f) separating the immobilized third complex from the CD8⁺ cells, thereby isolating the CD8⁺ cells.

2. The method of claim 1, wherein the CD8⁺ cells are human CD8⁺ cells.
3. The method of claim 1, wherein the first antibody is a monoclonal antibody.
4. The method of claim 3, wherein the monoclonal antibody is produced by a hybridoma cell line selected from the group consisting of the cell line designated 37B1 (ATCC Accession No. HB-12441) and the cell line designated 8G6 (ATCC Accession No. HB-12657).
5. The method of claim 1, wherein the immobilized second antibody comprises an antibody operably affixed to a magnetic bead.
6. The method of claim 1, wherein the agent which causes the dissociation of immobilized third complex is the polypeptide designated CD8-3 and having the amino acid sequence AAEGGLDTQRFSG.
7. A hybridoma cell line which produces a monoclonal antibody which specifically binds to CD8 molecules present on the surface of CD8⁺ cells but does not activate the CD8⁺ cells.
8. The hybridoma cell line of claim 7, wherein the hybridoma cell line is selected from the group consisting of the cell line designated 37B1 (ATCC Accession No. HB-12441) and the cell line designated 8G6 (ATCC Accession No. HB-12657).

9. The monoclonal antibody produced by the hybridoma cell line of claim 7.

10. The monoclonal antibody produced by the hybridoma cell line of claim 8.

11. A polypeptide useful for generating the monoclonal antibody of claim 9 which comprises the amino acid sequence AAEGLDTORFSG.

12. The polypeptide of claim 11, wherein the polypeptide is the polypeptide designated CD8-3 and having the amino acid sequence AAEGLDTORFSG.

13. A population of CD8⁺ cells isolated by the method of claim 1.

14. A kit for use in isolating CD8⁺ cells which comprises, in separate compartments,

(a) an antibody which specifically binds to the sequence AAEGLDTORFSG, or portion thereof, on CD8 molecules present on the surface of CD8⁺ cells, but does not activate the CD8⁺ cells once bound thereto; and

(b) an agent which causes the dissociation of a CD8⁺ cell-antibody complex.

15. The kit of claim 14, wherein the agent which causes the dissociation of a CD8⁺ cell-antibody complex is the polypeptide having the sequence AAEGLDTORFSG.

[illegible]

Abstract of the Disclosure

10

15

As a below named inventor, I hereby declare that:

I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled Method Of Isolating CD8+ Cells, And Related Hybridoma Cells, Antibodies And Polypeptides, the specification of which

(check one) ☒ is attached hereto.

□ was filed on _____ as

Application Serial No. _____

and was amended on _____.
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s):

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119	
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

60/124,253
(Application Number)

March 12, 1999
(Filing Date)

(Application Number)

(Filing Date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No. Filing Date Status

Application Serial No. Filing Date Status

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith as well as to file equivalent patent applications in countries foreign to the United States including the filing of international patent applications in accordance with the Patent Cooperation Treaty: Audley A. Ciamporzero, Jr. (Reg. #26,051), Steven P. Berman (Reg. #24,772), Andrea L. Colby (Reg. #30,194), Michael Stark (Reg. #32,495), and Alan J. Morrison (Reg. #37,399) One Johnson & Johnson Plaza, New Brunswick, NJ 08933.

09321527-030800

